Applicants have resubmitted each section of previously filed amendments to the specification on separate pages.

Respectfully submitted,

Date: October 7, 2004

Barbara S. Kitchell, Registration No. 33,928
Attorney for Applicant
EDWARDS & ANGELL, LLP
P.O. Box 55874
Boston, MA 02205
(203) 353-6848

Customer No.: 21874

[0068] ~~*Fig. 5* is a perspective view of wild type plants (WT) versus representative transgenic plants overexpressing AVP1 (AVP1-1 and AVP1-2) grown in salty soil.~~ Five wild-type plants (WT) and two AVP-1 overexpressing transgenic lines (AVP1-1 and AVP1-2) were grown on soil in a 10 hour light/dark cycle. Plants were watered with a diluted nutrient solution (1/8 MS salts) for six weeks and subsequently watered with a diluted nutrient solution supplemented with NaCl. The concentration of NaCl began with 100 mM and was increased every four days by 100 mM. The photograph in ~~*Fig. 3*~~ *Fig. 1A* corresponds to plants at the tenth day in the presence of 300 mM NaCl. *Fig. 3* illustrates that the two AVP-1 plant types (aVP1-1 and AVP1-2) were significantly hardier in salty soil as compared to wild-type plants. The fact that genetically engineered *Arabidopsis thaliana* plants that overexpress either AVP1 (the pyrophosphate-energized vacuolar membrane proton pump, this work) or AtNHX1 (the Na^+/H^+ antiporters, (Apse, M., *et al., Science, 285*:1256-1258 (1999)) and this work) are capable of growing in the presence of high NaCl concentrations strongly supports the strategy described herein. A double transgenic plant would be expected to demonstrate a further enhanced salt tolerant phenotype. These *Arabidopsis thaliana* transporters or their counterparts may perform similar function in important agricultural crops. *Fig. 5* is a drawing of wild type plants (WT) versus representative transgenic plants overexpressing AVP1 (AVP1-1 and AVP1-2) grown in salty soil.

[0072] Two transgenic lines of *Arabidopsis thaliana* were analyzed, *AVP1-1* and *AVP1-2*. Each line contains extra copies of the *35S:AVP1* gene inserted at a single chromosomal location. Analysis of AVP1 protein levels in membrane fractions isolated from shoots ~~show~~ shows that these transgenic plants express more AVP1 protein than does the wild type *(AVP1- 1*, 1.6 fold and *AVP1- 2*, 2.4 fold increase over wild type, P-value = 0.0005) ~~(Fig. 1)~~ (Fig. 4) as determined from four independent immunoblots ~~Western blots~~. The differences between these transgenic plants could be due to the number of copies of *AVP1* inserted into the genome or the sites of insertion. The transgenic plants overexpressing AVP1 are more salt tolerant than wild type plants (Figs. 2 and 3). Plants from both *AVP*1-1 and *AVP*1-2 transgenic lines grow well in the presence of up to 250 mM NaCl whereas wild type plants grow poorly and exhibit chlorosis. After 10 days in these conditions wild type plants die, whereas the transgenic plants continue to grow well.

[0073] The enhanced tolerance to salinity and drought in transgenic plants with increased levels of AVP1 is most easily explained by an enhanced uptake of toxic cations such as sodium into the vacuole. Presumably, the greater AVP1 activity provides increased H^+ to drive the secondary active uptake of cations into the lumen of the vacuole (Fig. ~~2C~~ 6C). If so, there must be a compensatory transport of anions to maintain electroneutrality. The resulting vacuolar solute content would confer greater water retention, permitting plants to survive under conditions of low soil water potentials. Furthermore, at high Na+ concentrations, the increased H+ gradient could also enhance the driving force for At NHX1-mediated Na+/H+ exchange, thereby contributing to the Na+ sequestration into the vacuole of *AVP*1 transgenic plants. Presumably, any toxic effects intrinsic to Na+ are mitigated by this sequestration in the vacuole. This scenario predicts that a transgenic plant engineered to overexpress both, the AVP1 H+-pump and the ATNHX1 Na+/H+ angtiporter would tolerate even higher NaCl stresses than *AVP*1 single transgenic plants.

[0074] Fig. ~~6~~ 6A and 6B show ~~is~~ a graph of Na^+ and K^+ content of wild-type plants (WT) versus representative transgenic plants overexpressing AVP-1 (1' and 2') grown in salty soil. Five wild-type plants (WT) and two AVP-1 overpexressing transgenic lines (1' and 2') were grown on soil in a 10 hour light/dark cycle. Plants were watered with a diluted nutrient solution (1/8 MS salts) for six weeks and subsequently watered with a diluted nutrient solution supplemented with NaCl. The concentration of NaCl began with 100 mM and was increased every four days by 100 mM. The photograph corresponds to plants at the tenth day in the presence of 300 mM NaCl. Parts of the plant above ground were harvested after 24 hours in the presence of 200 mM NaCl and their fresh weight measured. After 48 hours at 75°C, the dry weight was measured. Na+ and K+ content was determined by atomic absorption. Values in the graphs of Fig. 4 are the mean +/- SE (n = 4). As can be seen from the graphs Na+ and K+ content in the transgenic lines (1' and 2') was significantly higher than that of wild-type counterparts.

[0076] The above data ~~is~~ are consistent with the hypothesis that transgenic plants overexpressing AVP-1 have an enhanced H+ pumping capability at their tonoplast and that an enhanced H+ supply results in greater ion accumulation in the vacuole through the action of H+-driven ion transporters. To further support this theory, CA++ uptake capability of wild-type and transgenic vacuolar membrane vesicles was determined.

[0077] It is well documented that Ca^{++} enters the plant vacuole via a Ca^{++}/H^{+} antiporter (K. S. Schumaker, H. Sze, *Plant Physicol.* **79**, 1111-1117 (1985)). Furthermore, the genes encoding the Arabidopsis thaliana Ca++/H+ antiporters CAX1 and CAX2 have been isolated and characterized (K. D. Hirschi, R.-G. Zen, K. W. Cunningham, P. A. Rea, G. R. Fink, *Proc. Natl. Acad. Sci. USA* **93**, 8782-8786 (1996)). ~~Fig. 8~~ Fig. 7 shows that Ca^{++} uptake in the 35SAVP-1 transgenic vacuolar membrane vesicles is 36% higher than it is in vesicles obtained from wild type. Application of the Ca++ ionophore A23 lowered the 45Ca++ counts to background levels demonstrating the tightness of the vesicles (Fig 8)) K. S. Schumaker, H. Sze , *Plant Physiol.* **79**, 1111-1117 (1985)).

[0080] Transgenic plants that overexpress a vacuolar proton-pumping pyrophosphatase, such as for example AVP 1, also produce an increased yield of seeds. Referring to ~~Fig. 1,~~ Fig. 9, the seed yield, as expressed by the weight of seeds produced, is higher for AVP1-1 transgenic plants as compared to wild-type plants. This increased seed yield is a result of the pollen from the transgenic plant having an enhanced ability to fertilize, referred to herein as fertilization competence.

[0081] To demonstrate that the improved seed yield is a result of the improved fertilization competence of the pollen from the transgenic plant, the pollen from wild-type *Arabidopsis thaliana* plants was used for pollination of two lines of transgenic *Arabidopsis thaliana* plants transformed to overexpress AVP 1 (these two lines of transgenic plants are referred to herein as AVP 1-1 and AVP 1-2). Referring to Figs. ~~2A and 2B,~~ 10A and 10B, the transgenic plants, pollinated with pollen from wild-type plants produced an average of between about 15 and 20 seeds, with an average seed pod mass of between about 2.5 and 3 milligrams. These results were compared to the seed yield obtained when pollen from the two lines of *Arabidopsis thaliana* plants was used to pollinate wild-type *Arabidopsis thaliana* plants. Referring again to Figs. ~~2A and 2B,~~ Figs. 10A and 10 B, the wild-type plants fertilized with transgenic pollen produced an average of between about 30 and 35 seeds, with an average seed pod mass of between about 4 and 5 milligrams.

[0083] Similar results have also been observed in other plant species transformed to overexpress a vacuolar proton-pumping pyrophosphatase. Referring to ~~Fig. 3,~~ Fig. 11, the volume of seeds produced by wild-type tobacco plants is compared to the seed pod volume produced in transgenic tobacco plants transformed to overexpress a vacuolar proton-pumping pyrophosphatase. The volume of five seed pods from each plant was weighed. For the wild-type tobacco plants, the volume of seeds in five pods was between about 0.5 milliliters and 0.8 milliliters. For the three lines of transgenic tobacco plants tested, the volume of seeds in five pods was between about 1.2 milliliters and 1.4 milliliters. The transgenic tobacco lines were crossed and the volume of five seed pods was measured. The volume of five seed pods from the crossed lines of tobacco plants remained between about 1.2 milliliters and 1.4 milliliters (see Fig. 11).